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14. **ABSTRACT**
The purpose of this research project is to identify genes that are hypermethylated in epithelial ovarian cancers on a genome-wide scale and determine if this is histology-specific. The approach is to use normal ovarian surface epithelium (NOSE) and malignant cells obtained directly from surgically removed specimens in order to most closely approximate the methylation status in vivo. Cultured cells are mock treated, or treated with 5-AzaC, a potent DNA methyltransferase inhibitor, followed by microarray analysis to identify genes that exhibit increased expression in response to drug treatment. The methylation status will be confirmed in the original tumor and assessed in a large panel of ovarian cancer specimens to determine prevalence of aberrant methylation. We will analyze five cancers each of serous, endometrioid, mucinous and clear cell histologies, along with five NOSE specimens. We have developed protocols for culture and 5-AzaC treatment of normal and malignant ovarian cells and criteria (e.g., ≥90% epithelial, RNA quality) for microarray analysis. We have accrued 13 malignant samples (3 serous already arrayed, 1 serous and 1 endometrioid pending) and 19 NOSE specimens (9 that meet our criteria). Because of small cell numbers, NOSE specimens will be pooled for array hybridization. Collection of cancer and NOSE samples is ongoing, and analysis of candidate genes will begin once we obtain initial array results for the pooled NOSE controls.

15. SUBJECT TERMS
Ovarian cancer, epigenetics, methylation, epigenetic predisposition, microarrays

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INTRODUCTION

The purpose of this research project is to identify genes on a genome-wide scale that are epigenetically deregulated in ovarian cancer. The approach is to culture and expand primary ovarian cancer cells of different histologies and in sufficient numbers to enable treatment with a chemical inhibitor of DNA methyltransferase enzymes followed by microarray analysis to identify genes that exhibit increased expression in response to drug treatment. A subset of these genes will be examined for changes in promoter region methylation status to verify that they were indeed epigentically silenced in the original cancers. This information will then be used to determine the prevalence of that epigenetic deregulation in a larger sample of frozen ovarian cancer specimens. Finally, analysis of the array data obtained from epithelial ovarian cancers of different histologies will be performed to determine whether there are epigenetic signatures that distinguish tumor histology.

BODY

Over the past funding period, we have made significant progress toward the aims of the proposal. A summary of the work accomplished thus far is detailed below.

Task 1. Optimize treatment of cells with 5-AzaC.

We have optimized 5-azaC treatment of the cultured cells obtained from primary malignancies and the normal ovarian surface epithelium (NOSE) cells. This was initially done using established ovarian cancer cell lines and 5-AzaC-induced demethylation was confirmed for the *ARHI/NOEY2* locus, which is imprinted and normally differentially methylated [1] but becomes hypermethylated in ovarian [2] and breast [3] cancers. We also utilized the methylation status of *MAL*, another gene that we have shown is differentially expressed between long and short term survivors of ovarian cancer [4] and that we have subsequently identified as being aberrantly methylated in ovarian cancers (unpublished data). Based on dose-response and time course experiments, we established that treating cells at ~60-80% confluency for 72 hours with 5 μM 5-AzaC gave optimal results.

Culture of cells from primary ovarian malignancies. We have established criteria and a protocol for culture of malignant cells obtained from tumor debulking. Acquisition of appropriate malignant tissues for drug treatment and microarray analysis has been slower than anticipated, largely because we require pure histology and >90% cytokeratin-positive cells at time of harvest (post-treatment) in order to qualify for micorarray analysis. Table 1 lists the specimens that have been collected and the outcome. The final pathology reports are not obtained until after the cultures have been initiated, so in several cases the histologies do not meet our criteria (e.g., DoD-OC-2, 3 and 4). We nevertheless have treated these cultures and have frozen the cell pellets for later use in these studies when we are assessing response of specific genes to 5-AzaC treatment.

Our standard protocol involves first growing the cells to confluency after cell dispersion in culture medium and then a brief trypsinization to remove fibroblast cells prior to trypsin-induced detachment of the remaining cells in the plates [5]. Two plates each are used for no treatment, mock treatment, or 5-AzaC treatment. A portion of the untreated cells are frozen at harvest as “passage 1” and slides are prepared for cytokeratin staining from the untreated dishes. Micrographs have been taken for the untreated, treated, and mock-treated cells at the time of harvest to help document cell phenotype and morphologic characteristics. Cell pellets are

frozen at -80°C pending confirmation of the percent epithelial component by cytokeratin staining. Once verified, RNA is prepared from the mock treated and treated cell pellets for RNA quality and integrity checks.

Primary Malignancies			
Sample Number	Histology	Cytokeratin	Microarrayed
DoD-OC-1	Serous	>99%	Yes
DoD-OC-2	Serous Borderline	>90%	No
DoD-OC-3	Poorly differentiated	ND	No
DoD-OC-4	Poorly differentiated	>90%	No
DoD-OC-5	Serous	>90%	Yes
DoD-OC-6	Serous	>90%	Yes
DoD-OC-7	Endometrioid	>90%	Ready
DoD-OC-8	Serous	~80%	No
DoD-OC-9	Unknown	ND	No
DoD-OC-10	Serous	Pending	Ready
DoD-OC-11	Serous	in culture	
DoD-OC-12	Serous	in culture	
DoD-OC-13	Pathology pending	in culture	
Primary NOSE			
Sample Number	Growth	5-AzaC treatment	Microarrayed
NO-1	Contaminated	No	No
NO-2	No	No	No
NO-3	Poor	No	No
NO-4	No	No	No
NO-5	Yes	Yes	Pending
NO-6	Yes	Yes	Pending
NO-7	Yes	Yes	Pending
NO-8	Yes	Yes	Pending
NO-9	No	No	No
NO-10	No	No	No
NO-11	Yes	Yes	Pending
NO-12	Yes	Yes	Pending
NO-13	Yes	Yes	Pending
NO-14	No	No	No
NO-15	Yes	Yes	Pending
NO-16	No	No	No
NO-17	No	No	No
NO-18	Yes	Yes	Pending
NO-19	Contaminated	No	No

Culture of normal ovarian surface epithelium cells. Since the NOSE cells are derived from the single layer of cells on the surface of the ovary and are obtained by gentle intraoperative scraping, there are very few cells from which to initiate cultures that grow to sufficient numbers for microarray analysis. We have therefore opted to use pooled samples of NOSE for the microarray analysis. The procedure for growing the scraped surface epithelial cells is as follows:

Fresh ovarian surface epithelial cells scraped prior to oophorectomy are transferred to a sterile 50-ml conical tube containing saline. The cells are immediately transferred to the laboratory where they are pelleted by low-speed centrifugation. The pelleted cells are then resuspended in 6 ml of a1:1 preparation of Medium 199:MCDB-105 with 15% FBS. The cell suspension is then divided into thirds: one third is frozen (after pelleting) for future isolation of nucleic acid and the other two thirds are equally divided between two 3.5 cm dishes. The cells are cultured until attached and they begin to form colonies. Typically, after about 10-15 days, visible colonies have begun to form and at this point one dish is mock treated and the other treated with 5-AzaC. The cells are incubated for 72 hours followed by harvest of cell pellets and freezing at -80°C. As shown in Table 1, we now have nine NOSE specimens that have been treated or mock treated, stored and are ready for RNA isolation and microarray analysis. Depending on the yield of RNA from each specimen, we will pool samples until ~4 µg of total RNA is obtained since hybridization to the genechips requires ~3 µg of total RNA.

Task 2. Testchip analysis.

For the three primary ovarian cancer cells we have performed microarray analysis on thus far, all were shown to have good RNA integrity and quality using the Agilent Bioanalyzer through the Duke Microarray Facility. We will continue use of this analysis prior to all microarray hybridizations.

Task 3. GeneChip U133A Plus 2.0 analysis of five 5-azaC treated primary cultures of normal OSE and five serous ovarian carcinomas.

We have performed Affymetrix U133A Plus 2.0 on three serous epithelial ovarian carcinoma specimens thus far, and have several others pending.

We are just at the point where we feel we have a sufficient number of treated NOSE specimens that we can perform the first microarray hybridization for NOSE (see Table 1 for a list of the NOSE specimens thus far obtained and their outcome). We had originally proposed to array five independent NOSE samples; based on our pooling strategy we plan to continue to accrue these specimens with an aim toward arraying five *pooled* NOSE samples, each pool representing multiple individually treated NOSE specimens, depending on the yield of RNA obtained from each sample as described above.

Task 4. Validation of gene hypermethylation status.

This task has not yet been undertaken because of the inability to compare the microarray results from the cancer specimens to those from the NOSE. We will begin this analysis once we have data from the first pooled NOSE samples. In the meantime, we have worked with Dr. Terrence Furey, a computational biologist here at Duke and one of the developers of the UC Santa Cruz Genome Browser [6], who has annotated all of the gene probes on the Affymetrix U133Plus 2.0 arrays for association with promoter CpG islands that are located within 1.5 kb of the annotated transcription start site. This will facilitate our analysis of the data obtained by allowing us to immediately determine whether a given gene of interest is in proximity of a CpG island.

Task 5. Perform 5-azaC treatment and GeneChip U133A Plus 2.0 analysis of five each clear-cell, mucinous, and endometrioid tumors.

We have successfully treated and confirmed the epithelial nature of one endometrioid ovarian cancer specimen and are ready to submit RNA for quality and integrity checks and microarray hybridization if the RNA is of sufficient quality.

Task 6, Determine methylation status of the genes in N=43 normal ovaries that are hypermethylated in ovarian cancer.

This task was related to specific aim 3 of the grant, which was omitted per the DoD review.

KEY RESEARCH ACCOMPLISHMENTS

- Optimization of 5-azaC treatment completed; standard protocol has been established.
- Gene reactivation was confirmed in ovarian cancer cell lines.
- Protocol for culture of primary normal ovarian surface epithelial cells established.
- Ongoing collection and 5-azaC treatment of NOSE cells from surgery.
- Criteria for primary cancer specimen collection established, ongoing collection.
- RNA check and microarray hybridization (Affymettrix U133 Plus 2.0) has been performed for three serous epithelial ovarian cancer specimens.
- Annotation of genes containing promoter CpG islands was performed for the Affymetrix Array probe sets.
- One endometrioid cancer and a fourth serous cancer have been treated. The serous specimen is awaiting confirmation of the epithelial component of the cultured cells prior to array analysis and the endometrioid specimen has been confirmed.

REPORTABLE OUTCOMES

There are no reportable outcomes at this time.

CONCLUSIONS

We have made good progress toward the tasks outlined in the Statement of Work and are now focused on acquisition of patient samples for treatment and microarray work. We will begin analysis of the data generated once we have also obtained microarray data from at least one pooled NOSE sample for appropriate comparisons to the malignant specimens. As expected, accrual of the other less common malignancies including those of endometrioid, mucinous, and clear cell histologies will likely take an extended period of time, given their rarity and the strict criteria they must meet for microarray analysis. If we are not making steady progress for one or more of the histologic types by the end of the second year of this project, our alternative plan is to increase the number of serous epithelial cancers we analyze which will help to provide a stronger statistical basis for identifying genes that are targeted for hypermethylation in these malignancies.

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APPENDICES

N/A